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Patentanmeldung Nr. Patent application No. Demande de brevet n°

99113251.5

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# Blatt 2 der Bescheinigung
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# Delivery system for biological material

The present invention relates to a composition and method for delivery of biological material, especially nucleic acids into target cells and/or into the nucleus.

The delivery of biological material raises many promising opportunities to treat diseases especially the delivery of DNA for the treatment of both genetic and infectious origins. It is based on delivering biologically active substance as peptides, proteins or genes into somatic cells of an organism in order to influence the cell metabolism or switch off defective genes, or to replace a defective gene with an intact gene, or to enable these cells to form a protein that possesses a prophylactic or therapeutic effect.

Currently, viruses comprise the most popular vectors for in vivo gene delivery, particularly with the use of improved DNA packaging techniques. The principle underlying this technology is that parts of the viral genes are replaced by the desired gene with which the virus then reproduces.

However, viral vectors can give rise to problems when being used in living organisms. There is a danger of recombination with wild-type viruses of the same species, and viral coat proteins can trigger immune reactions in the recipient. A further disadvantage of viral vectors is that the geometry of the viruses restrict their ability to accommodate many foreign genes of interest.

Various non-viral delivery systems have been developed and tested. These systems include liposomes, monocationic or polycationic lipids as well as cationic polymers.

For successful application the DNA delivery system must be small enough to gain access to target cells. This frequently involves extravasation through endothelia, and the hyper-permeable endothelia associated with tumors have a size restriction of about 70nm (Ref. 2). In addition, most forms of triggered membrane penetration act via the endosomal membrane following endocytosis and pinocytic internalisation is usually limited to materials of less than 100 nm diameter (Ref.3).

Given the large size of Biomolecules, especially of DNA expression vectors in free solution, it is advantageous for the DNA to be compressed. It is known that DNA can

be condensed into polyelectrolyte complexes simply by the addition of polycations such as polylysine (PLL) , although the resulting particles are probably too large (80 - 100 nm diameter) for efficient access and entry into cells (Ref 4).

It is also known that conjugates containing higher molecular weight (MW) PLL clearly have on average a larger size and greater polydispersity than those containing lower molecular weight PLL. For example, conjugates based on the largest PLL (224500 Da) show a broad polydispersity of size, ranging up to maximum diameters of 300 nm, while conjugates based on the smallest PLL ( 3970 DA) show a small size and relatively uniform distribution (diameter ranging from 20-30 nm).

However, polycations are known to exert a range of non-specific toxicity effects and the concentration of electrostatic charges resulting from polyelectrolyte condensation could yield particles with extremely high charge density and possibly even increased toxicity.

The conjugates formed using higher molecular weight PLL show considerably greater cytotoxicity than those formed with the lowest molecular weight polycations. At present, the most popular cationic polymer is polyethylenimine (PEI) (Ref. 6). PEI -DNA complexes with different ratios of PEI nitrogen to DNA phosphate (N/P ratio) have been prepared and tested in a variety of in vivo models. Earlier experiments carried out with the branched 25kDa PEI show this polymer to be toxic, causing death within a few minutes, even when used at low N/P ratios. Better results are obtainable with linear polymers with a mean molecular weight (MW) of 22 kDA (Exgene 500). When complexing a reporter gene (pCMV-Luc) with Exgene 500 at ratios of 3 to 5 (N/P), transgene expression may be found 24h later in lungs, heart , spleen, liver kidney and brain (Ref. 6). However, toxic and immunogenic characteristics may not be overcome with the use of PEI.

Electroporation, another non-viral delivery system, is used to deliver biological material into target cells by applying an electrical field as described in U.S. Patent. No. 4,849,355; and U.S. Patent. No. 5,232,856. To deliver biological material into cells electric pulses are applied to target cells f.e. in a cell-suspension. The biological material in the suspension may diffuse into the cell through small pores, which are formed in the cell membrane by the application of the electric pulses.

Liposomal techniques have been combined with electroporation techniques to encapsulate the biological materials in liposomes and fuse the liposomes with targeted cells by electrofusion in order to achieve higher efficiency delivery.

However, the liposome are weakly loaded and do not fuse well with the target cell in the electrical field.

In the US-Patent No 5,789,213, fully incorporated by reference, an electroporation system is described that relates to the use of a two phase polymer system that concentrates biological materials with the target cells, such that the materials can be introduced into target cells during and after administration of an electric pulse by concentrating both the target cells, and biological materials to be loaded, into one of the two phases.

A two-phase polymer method is capable of separating or partitioning cells, proteins and minerals (described in U.S. Pat. No. 4,181,589; and Partitioning in Aqueous Two-Phase Systems, 1985, eds., H. Walter, D. Brooks, and D. Fisher, publs. Academic Press wherein polymer concentrations are % w/w unless noted otherwise). The partition of particles into different polymer phases depends on the interfacial energy of the particles and the polymer solutions. By varying the interfacial energy governed by the polymer and salt concentrations, selected particles (cells, macromolecules) can be driven into a given phase, hence achieving the purpose of separation or partitioning by the use of combinations of polymers

According to US-Patent No 5,789,213 a composition is used which functions to concentrate both target cells and biological materials into a single phase, and function to reduce the volume of this phase by osmotic control so that cells and biological materials are encapsulated in this single phase in a concentrated form during electroporation. Biological materials are then driven into the target cells during electroporation, and subsequent colloidal–osmotic swelling of cells after electroporation is limited, result in a higher loading efficiency. For example a two-phase polymer system using polyethylene glycol (PEG; molecular size (m.w,) 8,000 (in daltons)) and one of three formulations of dextran (dx; m.w. 9,000, and 71,000 and 249,000) is described.

However, electroporation beyond other disadvantages requires special equipment and is limited to use in vitro. Therefore there is a strong need for an in vivo system with a high delivery efficiency. It is therefore an object of the invention, to provide an efficient delivery system for biological material, especially for polynucleotides in vivo and in vitro.

The problem is solved by a composition and a method according to the independent claims.

The dependent claims cover the preferred embodiments of the invention.

The invention is based on the finding, that under certain conditions an emulsion, especially a water in water emulsion, will spontaneously separate into a two phase polymer system and subsequently lead to the formation of microparticles with high transfection properties which can be used to introduce genes into a number of organs in vivo. The microparticles include a major fraction of the polynucleotides of the emulsion and can be used for delivery into a target cell even in vivo.

A better understanding of the features of the present invention will be obtained from the following description of preferred embodiments and examples.

One of the most popular cell transfection methods is the calcium phosphate precipitation method. For the use of the water evaporation from aqueous Dx -PEG system it is desirable to improve the calcium phosphate precipitation method which may be achieved by taking advantage of the phenomenon of incompatibility of such polymers as dextran (Dx) and polyethylenglycol (PEG) in aqueous solutions and the partitioning of particles and molecules in an aqueous two phase systems may be used.

A spontaneous phase separation is achieved when the water is partially evaporated from the monophase Dx-PEG-water system, and two aqueous phases are formed , in form of the water in water emulsion ( W1/W2, W1-dispersed phase, W2 -continous phase). The first phase consists of water and Dx (DxP) , the second phase consists of water and PEG (PEGP). Accumulation of DNA occurrs when the dispersed phase is DxP.

When conducting the separation phase process under the conditions of the calcium phosphate precipitation method, microparticles are formed. The mean diameter of the microparticles varies between 0,03 and 0,2 microns, the particle size is dependent on the DxP / PEGP volume ratio and evaporating rates. A DNA including grade of 0,3 -0,5 may be achieved. To improve the including grade, Dx substituted by DNA-binding proteins and peptides or oligonucleotides and components such as glucose phosphate, lactose phosphate, and others may be added.

For the preparation of the microparticles, lower concentrations of natrium phosphate and calcium chlorid can be used than in the case of the calcium phosphate precipitation standard method. However, microparticle (microsphere) transfection activity is one order of magnitude higher. During the evaporation process, a high

concentration of Dx in DxP occurs and hydrogen bonds are probably formed which act as a stabilizing phenomenon for microparticles structures (Ref. 9).

DNA-binding proteins and peptides may be attached to the Dx using methods that are known to the person skilled in the art and described in Sezaki,H. , Hashida, M. ; Crit. Rev. Ther. Drug Carrier System, 1984, V.1, p.1-38;

Example 1: Preparation of microparticles

10 ml of aqueous solution containing 100 mg of Dx ( MW 500 kDa), 500 mg of PEG (MW 6kDa), 0,1 mg of polynucleotide in form of plasmid DNA (pCMV LacZ), 1 mg of glucose phosphate, 10 mg of calcium chloride , PBS (pH 7.4) are prepared and then the evaporating process is started (evaporating rate was 500 mg per 1hour). After 18 hours, the evaporating process is stopped and microparticles are formed. After that, 9 ml of water are added and PEG is removed (see Fig. 1). The microparticles are frozen and dried for storage.

Example 2: In-vitro Experiment

Microparticles (see Ex. 1) are introduced in a HELA cells culture medium (2 mg per 200 thousand cells in a 2 $cm^2$ well). Fluorescent-labeled dextran (FDx) is used in the preparation process for the aqueous solution (first step of Ex. 1). After a 6 hour incubation time with the microparticles, the cells are washed in PBS containing BSA and fixed for 30 min. at -20 C in 5% acetic acid in ethanol. Microscopy then reveals intranuclear and intracellular localization of microparticles for all cells (Fig.2.).

Example 3: In vivo experiment

Containing pCMV-LacZ microparticles are introduced in mice by intravenous injection in the tail (25 mg microparticles per animal). The mice are killed one week after the injection and sequentially perfused with 5mg/l heparine in saline, 2% paraformaldehyde, saline and a standard ß-galactosidase revealing solution ( 0,8 mg/ml X-gal). After perfusion, all the organs are dissected and ß-galactosidase revelation is continued for 48 hours by immersion in the X-gal solution at 30 C. After staining, the organs are embedded in parafin for histochemistry. Transgene (LacZ) expression is found in lungs, heart , muscles, liver and brain. When using FDx microparticles , intracellular and intranuclear localization of microparticles occurs.

Whereas the invention is described in detail, examples are included for illustration. Modifications of the present invention that are obvious to the man skilled in the art are intended to be within the scope of the patent claims.

For reasons of disclosure the following documents are fully incorporated by reference herewith.

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Schröder, U. ; "Crystallized Carbohydrate Spheres for Slow Release and Targeting" ; 1985, Methods in Enzymology, V.112, p.116-128;

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References for non-toxicity of Dextran :

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Reza Mehvar, Megan A. Robinson, James M. Reynolds, "Molecular Weight Dependent Tissue Accumulation of Dextrans: In Vivo Studies in Rats "; Journal of Pharm. Sciences, 1994, V.83, No.10, p.1495-1499;

Thoren, L. , Develop. Biol. Stand., 1981, 48, 157-167;

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Figure 1.: preparation of microsparticles.

Claims

1. A composition for delivery of biological material into a target cell comprising:

biological material,
a preparation of an aqueous polymer system on the basis of an emulsion with at least two compounds being incompatible in aqueous solutions,
said compounds being present in a concentration in water that leads to formation of a dispersed phase by one of said compounds,
said dispersed phase including microparticles in said aqueous solution.

2. A composition according to claim 1 wherein, the emulsion is a water in water emulsion.

3. A composition according to claims 1 or 2, wherein first and second compounds are a carbohydrate-based polymers or derivatives thereof.

4. A composition according to claims 1 or 2, whereinfirst compound is a carbohydrate-based polymer or derivative thereof and second compound is a polyaliphatic alcohol or derivative thereof.

5. A composition according to one of the claims 1to 4, wherein the carbohydrate-based polymer is dextran, or dextrin, or a methylcellulose based polymer, or a carboxymethyl cellulose-based polymer, or polydextrose, or chitin or chitosan, and/or starch, or hetastarch, or derivatives thereof, or naturally occurring polymers as zein, and pullulan, or derivatives thereof.

6. A composition according to claim 5, wherein one compound is substituted by a DNA-binding agent.

7. A composition according to one of the claims 4 to 6, wherein the polyaliphatic alcohol is polyethylene oxide, or a derivative thereof, or polyethylene glycol (PEG), or PEG-acrylate, or polyvinyl acetate, or a derivative thereof.

8. A composition according to one of the above claims, said composition comprising a surfactant or a derivative thereof.

9. A composition according to claim 8, wherein said surfactant is polyoxyethylene sorbitan ( Tween-20,40,60,80).

10. A composition according to one of the above claims, said composition comprising co-polymers or block co-polymers.

11. A composition according to claim 10 wherein said co-polymer is poloxamer or Pluronic L-64 or Pluronic F-68, or a derivative thereof.

12. A composition according to one of the above claims, said composition comprising polyvinylpyrrolidone (PVP)

13. A composition according to one of the above claims, wherein said biological material comprises Polynucleotides, or Vaccines (microbes, viruses), or Proteins, or Peptides, or derivatives thereof.

14. A composition according to one of the above claims, wherein said biological material comprises Cytokines or monoclonal antibodies

15. A composition according to claim 14, wherein said Cytokines comprise Interferones or Interleukines,

16. A composition according to claim 6, wherein said DNA binding agent is a peptide or a protein.

17. A composition according to claim 16, wherein said peptide is a low molecular weight polylysines.

18. A composition according to claim 16, wherein said protein is a histone.

19 A composition according to claim 5, wherein said dextran has a molecular weight from 4 kDa to 5000 kDa.

20. A composition according to claim 13, wherein said polynucleotide is DNA.

21. A composition according to claim 13, wherein said polynucleotide is RNA.

22. A composition according to claim 21, wherein said RNA is antisense.

23. A composition according to claim 7 wherein said is polyethyleneglycol has a molecular weight from 3 kDa to 20 kDa.

24. A method for preparation of microparticles with use of a composition according to one of the above claims, wherein the concentration of water for formation of microparticles is achieved by evaporation of water from a one-phase system leading to a phase separation.

25. A method according to claim 24, wherein said evaporating process has a duration between 0,1 and 50 hours.

26. A method according to claim 24 or 25, wherein said evaporating process is carried out at a temperature between 0° C and 50° C.

27. A method according to one of the claims 24 to 26, wherein said evaporating process is carried out under a pressure of 0,1 to 760 mm Hg p.

28. A method according to one of the claims 24 to 27 wherein said evaporating process is stopped when the water concentration within the system is between 5 to 80 %.

29. A method according to one of the claims 24 to 28, wherein the calcium phosphate precipitation method is used.

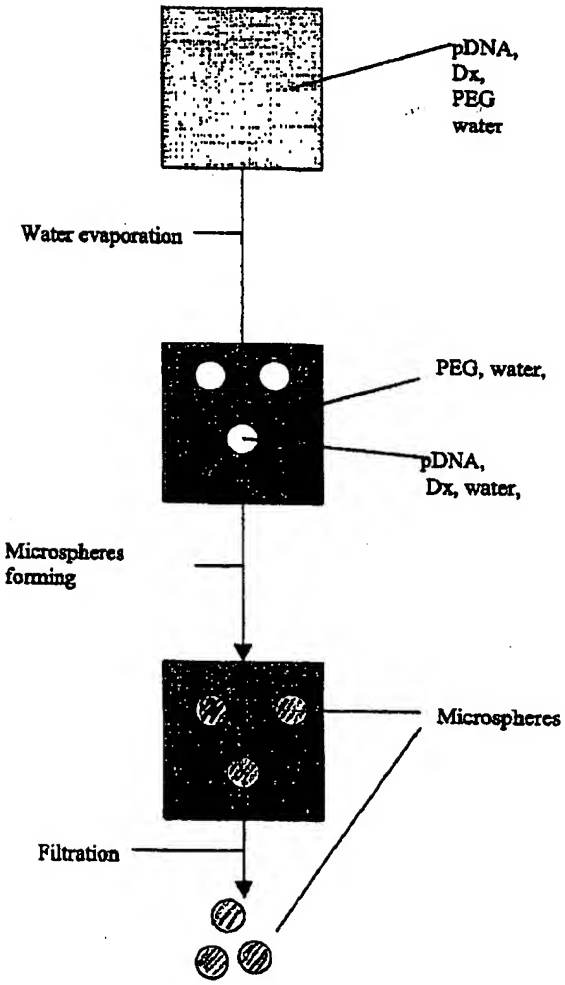


Fig. 1.
Preparation of microspheres

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# Delivery system for biological material

## Abstract

The present invention relates to a composition and method for delivery of biological material, especially nucleic acids into target cells and into the nucleus.